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Lebecetin, a C-Lectin Protein from the Venom of *Macrovipera lebetina* That Inhibits Platelet Aggregation and Adhesion of Cancerous Cells

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Abstract
A novel C-lectin protein, lebecetin, was purified and characterized from the venom of *Macrovipera lebetina*. It is a disulfide-linked heterodimer of 15 and 16 kD. The subunits are homologous to each other and to the other snake venom proteins of the C-type (Ca^{2+}-dependent) lectin superfamily. Lebecetin shows a potent inhibitory effect on whole blood and washed platelets induced by different agonists. It inhibits the agglutination of human fixed platelets in the presence of ristocetin. Lebecetin also interferes with the adhesion of IGR39 melanoma and HT29D4 adenocarcinoma cells. These two lines adhere to lebecetin used as matrix. Lebecetin is also able to strongly reduce IGR39 and HT29D4 cell adhesion to fibrinogen and laminin, but not to fibronectin and collagen types I and IV, respectively. Adhesion properties of lebecetin may thus involve integrin receptors.

Introduction

Lectins are defined as nonenzymatic carbohydrate-binding proteins, common in plants including trees, and particulary seeds. They have also been found in bacteria, roe, snails, vertebrates and mushrooms [1]. C-Lectin proteins (CLPs) have also been found in the venom of snakes belonging to the families of Elapidae, Viperidae and Crotalidae [2–4]. These proteins show an M_r of 30,000 D and exist as heterodimers linked by a single inter-

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chain disulfide bond. They share a structural homology of 30–70%. However, this group of Ca^{2+}-dependent lectin-related proteins exhibits different effects on blood coagulation and platelet aggregation. Several CLPs induce various effects on platelet functions by modulating the interactions between von Willebrand factor (vWF) and platelet GPIb. For example, botrocetin from *Bothrops jararaca* venom, binds to vWF and forms an activated complex that induces platelet agglutination [5]. Alboaggregins (A and B) from *Trimeresurus albolabris* venom [6, 7], echicetin from *Echis carinatus* venom [8] and agkicetin from *Agkistrodon acutus* venom [9] all bind to platelet GPIb and function as receptor blockers for vWF binding. However, alboaggregins induce platelet agglutination whereas echicetin and agkicetin inhibit platelet agglutination.

Lectin-like proteins were present in the venom of *M. lebetina*, mainly as dimeric proteins. A novel CLP, designated lebecetin, was isolated from *Macrovipera lebetina* venom and characterized as a potent platelet aggregation inhibitor with interesting adhesive properties.

Materials and Methods

Purification of Lebecetin

M. lebetina venom was dissolved in 0.2 *M* ammonium acetate (pH 6.8) and applied to a Sephadex G-75 column equilibrated with the same buffer. The fractions containing the main of antiplatelet activity were collected and then loaded on a cation exchange Mono S (HR5/5) column previously equilibrated with 50 m*M* HEPES/HCl, pH 7.5, and eluated with a linear NaCl (0–1 *M*) gradient. RP-HPLC (C8 column) was then used to purify the protein.

Amino Acid Sequence Analysis

Lebecetin was reduced and alkylated and separated into two subunits by RP-HPLC on a C8 column. The N-terminal sequence of each subunit was analyzed by automated Edman degradation (Model 476A, Applied Biosystem).

Platelet Aggregation Assay

Platelet-rich plasma (PRP) was prepared from rabbit blood by mixing whole blood with 0.1 vol of 3.8 of trisodium citrate followed by centrifugation at room temperature [10]. Platelet aggregation studies were carried out with a Chronolog aggregometer using 0.3 ml of PRP at 37 °C.

Platelets were isolated from human or rabbit blood using 5 m*M* EDTA as anticoagulant. Platelets were then separated from blood and washed twice with calcium-free Tyrode's buffer, pH 6.5, containing 0.1% glucose, 0.25% gelatin, 2 m*M* $MgCl_2$ and 0.2 m*M* EGTA as previously described [11]. The final suspension was made in a modified Tyrode's buffer without EGTA, pH 7.4. Platelet aggregation was monitored at 37 °C under stirring (1,100 rpm) in a Chronolog aggregometer. Platelets ($3 \cdot 10^8$ cells/ml) were incubated for 2 min with 2 m*M* $CaCl_2$ in the aggregometer cuvette in the presence of lebecetin before the addition of agonists.

Preparation of Formalin-Fixed Platelets

Fixed human platelets were prepared as previously described [12]. They were incubated with 2% of formalin in Tris-saline buffer overnight at 4 °C. Finally, they were washed twice with Tris-saline buffer. The final platelet pellets were suspended in an equal volume of Tris-saline buffer containing BSA (20 mg/ml).

The agglutination of fixed washed platelets induced by bovine vWF was measured using an aggregometer as previously described [11].

Cell Adhesion Assay

Flat bottom 96-well microtiter plates were coated for 2 h at 37 °C with 50 µl of one of the following purified extracellular matrix (ECM) proteins: fibronectin (10 µg/ml), vitronectin (10 µg/ml), laminin (2 µg/ml), fibrinogen (50 µg/ml), collagen type I (10 µg/ml) and collagen type IV (10 µg/ml). Coated wells were blocked with 1% BSA in PBS for 1 h. The melanoma cell line IGR39 and adenocarcinoma cell line HT29D4 were routinely cultured in Dulbecco's modified Eagle's medium (DME medium) containing 4.5 g/l glucose and 10% fetal calf serum in a humidified atmosphere of 5% CO_2. Cells were harvested in single cell suspensions by treatment with 0.53 m*M* EDTA in PBS, washed twice with DME medium containing 0.2% BSA (adhesion buffer) and resuspended in the same medium in the presence of lebecetin. After incubation for 30 min at room temperature, cells were added to coated wells in a volume of 50 µl (10^6 cells/ml) and allowed to adhere to the substrate for 1 h in a cell culture incubator. Unattached cells were removed by gently washing three

times with adhesion buffer. Residual attached cells were fixed by 1% glutaraldehyde, stained by 0.1% crystal violet and lysed with 1% SDS. The absorbance was measured at 600 nm by a microplate reader (model Σ960 from Metertech).

Results

Purification and Characterization of Lebecetin

Lebecetin was purified to homogeneity from the venom of *M. lebetina* in three steps: first by gel filtration chromatography, then by cation exchange chromatography and finally by RP-HPLC. Each fraction was assayed for inhibition of aggregation activity on rabbit whole blood. On SDS-PAGE (15%), lebecetin behaved as a molecule of 29 kD under nonreducing conditions and separated into bands of 15 and 16 kD under reducing conditions. Lebecetin (5 nmol) was reduced, alkylated and separated into two peaks by RP-HPLC on a C8 column. MALDI TOF spectrometry data confirmed results obtained by SDS-PAGE analysis and in particular the heterodimeric nature of lebecetin. The N-terminal amino acid sequence of both chains was determined and aligned with those of other members of C-type lectin-like protein from snake venom. α- and β-Subunits show 42% identity between them and respectively 28–67% [13, 14] and 40–55% [14, 15] identity with those of the other corresponding α and β CLP-related sequences.

Effect of Lebecetin on Platelet Aggregation

Lebecetin showed a potent inhibitory effect on platelet aggregation in rabbit whole blood induced by ADP (1 m*M*) and Ca^{2+}- ionophore (10^{-3} m*M*) with an IC_{50} of about 4 n*M*. This CLP was also able to inhibit rabbit and human washed platelet aggregation induced by thrombin (0.04 IU/ml). The IC_{50} was about 0.1 n*M*. Individually, both α- and β-subunits exhibited a distinct strong inhibitory effect. These results may suggest that individual lebecetin subunits contained interaction sites that enable them to bind to platelets.

Lebecetin inhibited both the aggregation of platelet-rich plasma induced by the antibiotic ristocetin and the agglutination of fixed platelets induced by bovine vWF in a dose-dependent manner. The concentration of lebecetin required for 50% inhibition of agglutination of the fixed platelets was approximately 0.5 n*M*. EDTA and EGTA significantly reduced the agglutination inhibiting activity of lebecetin. These results suggest the importance of Ca^{2+} for the expression of lebecetin activity.

Effect of Lebecetin on Cancerous Adhesion Cells

The inhibitory effect of lebecetin on the adhesion of cancerous cells to immobilized ECM (e.g., fibrinogen, collagen I and IV, laminin and vitronectin) was tested. Lebecetin potently inhibited the adhesion of HT29D4 cells (derived from adenocarcinoma) to laminin matrix as well as the adhesion of IGR39 melanoma cells to fibrinogen matrix. Moreover IGR39 cells and HT29D4 cells adhered tightly to immobilized lebecetin. The adhesion of lebecetin to IGR39 cells was dose-dependent with an ED_{50} of 30 μg/ml.

Discussion

Crude *M. lebetina* venom prevents platelet aggregation induced by collagen, thrombin or PAF acether with similar IC_{50} around 100 μg/ml [16]. Based on this observation, we isolated and described a potent inhibitor of platelet aggregation and agglutination named lebecetin. It is composed of two covalently linked subunits. Lebecetin has a high level of

homology with N-terminal amino acid sequences of CLPs. Thus lebecetin belongs to the CLP class of snake venom proteins.

Lebecetin inhibits platelet aggregation induced by different agonists such as ADP, Ca^{2+}-ionophore and thrombin. It is also able to inhibit PRP and washed fixed platelets in the presence of ristocetin. On the other hand, we found that lebecetin predominantly inhibits adhesion of HT29D4 to immobilized laminin and IGR39 to immobilized fibrinogen.

Work is in progress to determine the integrins on the surface of these cells interacting with lebecetin.

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